

AN EXAMINATION OF THE HEXANE EXTRACT OF FLUE-CURED TOBACCO INVOLVING GEL PERMEATION CHROMATOGRAPHY¹

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Abstract—Techniques have been investigated for separating the hexane extract of flue-cured tobacco into classes based on polarity and molecular size. The method developed, which depends upon counter-current distribution for polarity separation and gel permeation chromatography for molecular size separation, is reproducible, non-destructive, and permits quantitative recovery. Its applicability has been demonstrated by the isolation and identification of several compounds and classes of compounds by following it with conventional chromatographic techniques (column, thin-layer, and gas/liquid chromatography). Among the substances isolated were glycerides, solanesol esters, sterol esters (including cholesterol esters), solanochromene (plastochromenol-8), solanesol, α -tocopherol, β -amyirin, and sterols (including cholesterol). Molecular weight determinations indicated that 70 per cent of the non-basic hexane extract was below 800 in molecular weight.

INTRODUCTION

ACCORDING to a recent review² over 1200 compounds have been identified in tobacco and smoke. The hexane-extractable fraction has received considerable attention, primarily because of its suggested involvement with leaf quality and the generation of polynuclear aromatic hydrocarbons during leaf pyrolysis. In addition to the numerous studies of individual compounds or classes of compounds (see Ref. 2), Swain *et al.* reported a comprehensive study of this fraction in 1961.³ In this study, as well as others,^{4,5} a large portion (69 per cent) of the hexane extract was reported to consist of resinous material which could not be definitely characterized. Hellier developed a paper chromatographic, differential solubility technique which divided tobacco extracts into "soft" and "hard" resins.^{6,7} The latter resembled the resin fraction described by Swain *et al.*

An indication that the resin fraction had a high molecular weight³ suggested that it might be advantageous to separate the hexane extract by molecular weight differences before

¹ (a) A report of work done under Contract No. 12-14-100-7702(73) with the U.S. Department of Agriculture and authorized by the Research and Marketing Act of 1946. The contract was supervised by the Eastern Utilization Research and Development Division of the Agricultural Research Service. (b) Presented in part at the 21st Annual Tobacco Chemists Conference, 19-20 October 1967, Abstracts, p. 4. (c) For a preliminary report of some of this work see C. E. COOK, MARGARET E. TWINE and M. E. WALL, *Experientia* 23, 987 (1967).

² R. L. STEDMAN, *Chem. Rev.* 68, 153 (1968).

³ A. P. SWAIN, W. RUSANIWSKYJ and R. L. STEDMAN, *Chem. Ind.* 435 (1961).

⁴ R. A. W. JOHNSTONE and J. R. PLIMMER, *Chem. Rev.* 59, 885 (1959).

⁵ W. G. FRANKENBURG, *Advan. Enzymol.* 6, 309 (1946), quoted in Ref. 3.

⁶ D. N. HELLIER, *Chem. Ind.* 260 (1959).

⁷ W. W. REID and D. N. HELLIER, *Chem. Ind.* 1489 (1961).

RESULTS AND DISCUSSION

Extraction and Preliminary Separation

Table 1 outlines the procedure used for obtaining material. Continuous extraction with hot hexane, precipitation of acetone-insoluble substances, and removal of basic substances were followed by a short counter-current distribution so as to divide the extract into rough classes according to polarity. A relatively non-polar system (acetonitrile-hexane) was chosen on the basis of preliminary studies. If basic substances were not removed before the counter-current distribution step, they were found in the center tubes. All the steps in Table 1 could be carried out on a large scale, so the preliminary separation could be done quite efficiently.

Gel Permeation Chromatography

Initial experiments with polymer beads containing 2, 4, and 10 per cent divinylbenzene showed that best separation was achieved with the 2 per cent cross-linkage. Reproducibility was demonstrated by chromatographing several samples in sequence, when closely similar elution patterns were obtained. Since recovery was essentially quantitative a new sample could be applied before the first was completely eluted. Although sample size had to be limited to *ca.* 7 mg per g of dry polymer, the column size was readily scaled up with no loss in efficiency.

When all of the counter-current distribution fractions were chromatographed, the results shown in Table 2 were obtained. (Percentages in Table 2 are the average of several runs, average deviation $\pm 1-2$ per cent). In spite of the differences in polarity of material from the various CCD tubes, the contents of all the tubes were eluted within approximately the same volume of solvent. However, the more polar tubes contained relatively more low molecular weight material.

TABLE 2. GEL PERMEATION CHROMATOGRAPHY (GPC) OF THE COUNTER-CURRENT DISTRIBUTION (CCD) TUBES

CCD tube No.	GPC Fraction	Ave. M.W.	GPC tube Nos.	% of CCD tube	% of non-basics	% of hexane extract
0	A	2049	14-16	6	9	5.3
	B		17-18	8		
	C		19	3		
	D	934	20	3	9	5.3
	E		21-23	20		
	F		24	6		
	G	614	25-27	35	15.7	9.2
	H		28-30	11		
	I		31-35	8		
1	A	1596	15-17	2	0.6	0.4
	B		18	1		
	C		19-20	3		
	D	1091	21	2	1.0	0.6
	E		22-23	12		
	F		24	6		
	G	630	25-28	56	4.5	2.6
	H		29-30	11		
	I		31-36	11		

The number-average molecular weights¹⁰ in Table 2 show that the order of elution is always that of descending molecular weight and that the molecular weights for any given set of GPC tubes are closely similar. This is strong evidence that the gel column used fractionates by molecular size over the entire polarity range tested—a hypothesis borne out by the isolation of principal components in several of the fractions (see below). Thus the order of elution for tube zero is glycerides (M.W. 800–900), sterol esters (M.W. 600–700), free sterols (M.W. ca. 400).

It should be emphasized that the molecular weights determined for gel column fractions are average values, that they do not in themselves define the upper and lower limits of molecular weights in a mixture, and that the crude nature of the material renders them imprecise. Nevertheless it is instructive to consider their implications concerning the amounts of material in various molecular weight ranges. If we consider only the portion of the hexane extract which is non-basic and soluble in acetone, roughly 14 per cent has M.W. >1200, 16 per cent lies in the range 800–1200, 36 per cent in the range 450–800, and 35 per cent is lower than 450 in molecular weight. The highest range could be said to include “polymeric” materials; the second contains glycerides and solanesol esters plus some “polymers”. Molecular weights in the third range are of the order of solanesol, solanochromene, and sterol esters, and the lowest range should include such components as sterols, triterpenes, phenolics, and hydrocarbons.

These percentages would doubtless fluctuate with the type and previous history of the tobacco sample. None the less the results suggest that at least 70 per cent of the non-basic hexane extract from the sample examined consists of material in a size range amenable to structure determination by ordinary chemical and physical methods. The fact that percentages of identified compounds add up to a considerably lower figure (both in our hands and those of others) may be attributed to losses in purification—the difficulty of which is magnified by the multitude of compounds present in tiny amounts—and the presence of many unsaturated compounds which readily undergo autoxidation with a resulting increase in the resin fraction.

Thin-layer chromatograms of all the gel column fractions showed that marked simplification of adsorption chromatography had been achieved by the gel chromatography. Subsequent isolation of components was then carried out by either column or preparative thin-layer chromatography.

Identification of Components

I.r. spectra of 0-E, 1-E, and 2-C showed strong ester bands. The major component was isolated from 0-E by column chromatography and preparative TLC and identified as a mixture of glycerides by a color test for glycerol¹¹ and gas-liquid chromatography (GLC) of the acid components as the methyl esters.¹² The major acids were palmitic, linoleic, and linolenic, with lesser amounts of stearic and oleic, and minor quantities of myristic, capric, and unidentified acids.¹³ The glycerides of tobacco seed oil have received considerable study,⁴ with less attention given to leaf glycerides.⁶ The isolated glyceride fraction constituted over 1 per cent of the non-basic hexane extract. It seems likely that much of the other ester material

¹⁰ For a background discussion of the method used (which is actually a thermoelectric vapor pressure method) see R. U. BONNAR, M. DIMBAT and F. H. STROSS, *Number-Average Molecular Weights*, pp. 263–268, Interscience Publishers, New York (1958).

¹¹ R. D. SPENCER and B. H. BEGGS, *J. Chromatogr.* **21**, 52 (1966).

¹² A. RODGMAN, L. C. COOK and P. H. LATIMER, JR., *Tobacco Sci.* **3**, 125 (1959).

¹³ Cf. A. P. SWAIN and R. L. STEDMAN, *J. Assoc. Offic. Agr. Chem.* **45**, 536 (1962).

As a check against inadvertent contamination with cholesterol, a 1-kg sample of the tobacco was washed with benzene to remove surface contaminants before being carried through the extraction procedure. Solvents used were freshly opened reagent grade or distilled, and all steps were carried out by an operator wearing rubber gloves. Results were the same as before.

The presence of cholesterol in tobacco is not without precedent in the plant kingdom. Since its isolation from date palm pollen,^{23a} its presence has been inferred in other plants by mass spectrometry and/or gas chromatography.^{23b-h} The sterol fraction from saponification of tobacco tissue cultures was reported to contain *ca.* 1 per cent of a substance with the retention time and molecular weight of cholesterol, but positive identification was not made.²⁴ The present work provides rigorous identification. GLC analysis²⁵ indicated *ca.* 5 per cent of the sterol fraction was cholesterol.²⁶

CONCLUSIONS

The combination of counter-current distribution and gel permeation chromatography allows rapid fractionation of the non-basic hexane extract of tobacco into classes based on polarity and molecular weight. Both techniques are mild and permit complete recovery of material. In certain cases they form a convenient means of isolating a given substance. Thus the glyceride fraction is readily obtained almost pure after a simple silica gel chromatography, the sterol ester fraction is rapidly separated by a similar technique, and the main sterol fraction is partially crystalline and pure enough for direct GLC analysis of the sterols. These techniques may thus prove useful in comparison studies of different tobaccos and processing.

EXPERIMENTAL

Tobacco

A 1000-lb lot of Hicks variety flue-cured tobacco, U.S. government grade B4LV, harvested from just above mid-stalk (sixth of ten predicted primings) in late July, 1964, was kept in freezer storage. The crop was grown in Georgia on Tifton series soil. Fertilizer (4-8-12) was used at a rate of 1800 lb/acre, and MH-30 (maleic hydrazide) was used for sucker control.

Solvents

Solvents were carefully checked for non-volatile residue. Hexane for extractions was distilled before use unless it contained less than 10 mg of residue per l. Other solvents were reagent grade, but were also distilled unless obtained in glass bottles.

Extraction and Preliminary Fractionation (Table 1)

Tobacco (9.5 kg, ground in a ball and jewel mill) was soaked in 38 l. of hexane overnight and then extracted continuously with heating for 8 hr. Hexane was drawn off and 38 l. more added. Hot extraction was carried

²³ (a) R. D. BENNETT, S.-T. KO and E. HEFTMANN, *Phytochem.* **5**, 231 (1965). (b) M.-F. HUGEL, W. VETTER, H. AUDIER, M. BARBIER and E. LEDERER, *Phytochem.* **3**, 7 (1964). (c) P. DUPERON, W. VETTER and M. BARBIER, *Phytochem.* **3**, 89 (1964). (d) C. DJERASSI, J. C. KNIGHT and H. BROCKMANN, JR., *Chem. Ber.* **97**, 3118 (1964). (e) J. W. ROWE, *Phytochem.* **4**, 1 (1965). (f) E. HEFTMANN, E. R. LIEBER and R. D. BENNETT, *Phytochem.* **6**, 225 (1967). (g) R. D. BENNETT and E. HEFTMANN, *Archs Biochem. Biophys.* **112**, 616 (1965). (h) B. A. KNIGHTS, in *Gas Liquid Chromatography of Steroids* (edited by J. K. GRANT), p. 217, University Press, Cambridge (1967). Cholesterol percentages range from traces in some plants up to 11-12 per cent [in tomato (*Lycopersicon esculentum*) leaves and lemon (*Citrus limoni*) peel] of total sterols.

²⁴ P. BENEVENISTE, L. HIRTH and G. OURISSON, *Phytochem.* **5**, 31 (1966).

²⁵ A. ROZANSKI, *Anal. Chem.* **38**, 36 (1966).

²⁶ Since our preliminary report^{1b,c} appeared, B. RICHARDSON, J. R. BAUR, R. S. HALLIWELL and R. LANGSTON [*Steroids* **11**, 231 (1968)] have reported on the GLC analysis of sterols in tobacco, and suggest that the level of cholesterol may fluctuate.

supplied by F & M (split ratio *ca.* 50:1 to 200:1). The sample size ranged from 400–1000 μg in 20–50 μl of solution. Effluent samples were collected in Teflon tubes and rinsed into vials with CH_2Cl_2 . Hydroxy compounds (e.g. sterols) were often converted to the corresponding trimethylsilyl (TMS) or dimethylsilyl (DMS) ethers by heating for 0.25 hr on a steam bath with pyridine and either hexamethyldisilazane (for TMS ethers) or *sym*-tetramethyldisilazane (for DMS ethers). Methyl esters were analyzed on a 4 ft, 3 mm i.d. 10% EGSSX column (Applied Science Laboratories), with methyl pentadecanoate as an internal reference. Good separation was obtained at 180° for acids from C_{12} – C_{20} (the separation factor for stearic and oleic esters was 1.21).

Spectra

I.r. spectra were determined in CH_2Cl_2 , u.v. spectra in hexane or methanol on a Cary 14 Spectrophotometer, NMR spectra in CDCl_3 with internal tetramethylsilane standard on a Varian A-60 and mass spectra on a Perkin-Elmer-Hitachi (for which we thank Dr. Maurice Bursey of UNC-Chapel Hill), or an AEI MS-902 (Dr. David Rosenthal, Research Triangle Institute Regional Center for Mass Spectrometry).

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